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DOI: https://doi.org/10.1016/j.cbpa.2015.06.022

Comparative Biochemistry and Physiology A

Comparative methane emission by ratites: differences in food intake and digesta retention level out methane production

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Running head: Methane in ratites

Abstract

Ratites differ in the anatomy of their digestive organs and their digesta excretion patterns. Ostriches (*Struthio camelus*) have large fermentation chambers and long digesta retention, emus (*Dromaius novaehollandiae*) have a short gut and short retention times, and rheas (*Rhea americana*) are intermediate. A recent study showed that ostriches produce as much methane (CH_4) as expected for a similar-sized, non-ruminant mammalian herbivore. We hypothesized that emus and rheas produce less CH_4 than ostriches. We individually measured, by chamber respirometry, the amount of O_2 consumed as well as CO_2 and CH_4 emitted from six adult rheas (body mass 23.4 ± 8.3 kg) and two adult emus (33.5 and 32.0 kg) during 23-hour periods on a pelleted lucerne diet. In contrast to previous studies, which classified emus as non-producers, we measured CH_4 emissions at 7.39 and 6.25 L/day for emus and 2.87 ± 0.82 L/day for rheas, which is close to values expected for similar-sized non-ruminant mammals for both species. O_2 consumption was of a similar magnitude as reported previously. Across ratites CH_4 yield (L/kg dry matter intake) was positively correlated with mean retention time of food particles in the gut, similar to findings within ruminant species. In ratites, this relationship leads to similar body mass-specific CH_4 production for a high intake/short retention and a low intake/long retention strategy. Therefore, when investigating CH_4 production in herbivorous birds, it is advisable to consider various CH_4 measures, not only yield or absolute daily amount alone.

Key words: fermentation, herbivory, digestion, methanogenesis

1. Introduction

Animals differ in many characteristics of digestive physiology, including the amount of methane (CH_4) they emit per day (Crutzen et al. 1986; Miller and Wolin 1986; Jensen 1996). The best known example is the general difference between ruminants and non-ruminant herbivores (Franz et al. 2010; Franz et al. 2011), but why ruminants produce generally more CH_4 is not completely understood. Current explanations include general differences in the composition of the microbiome in the digestive tract (Jensen 1996; Morvan et al. 1996) or differences in the time that digesta is retained in the digestive tract (El Oufir et al. 1996; Goopy et al. 2014). In addition to this difference in the magnitude of CH_4 production, current concepts also include the possibility that some herbivore species are non-producers (Hackstein and Van Alen 1996).

Due to the enormous differences in their digestive tract anatomy and physiology, ratites are an interesting group of herbivores in this respect (reviewed in Frei et al. 2015b). Ostriches (*Struthio camelus*) have long paired caeca and a large colon, a capacious digestive tract, digesta retention times of a magnitude comparable to mammalian non-ruminant hindgut fermenters, and a moderate food intake level. Emus (*Dromaius novaehollandiae*) are characterised by a less capacious digestive tract without prominent colon and with short caeca, extremely short digesta retention times, and very high food intake levels. Rheas (*Rhea americana*) are intermediate, with capacious paired caeca but a short colon. Given the common concept that a long digesta retention time is required for a significant CH_4 production, ostriches would be expected to produce most, and emus to produce least, if any, CH_4. Consistent with this, CH_4 emission from the faeces measured in captive animals was higher for ostriches compared to rheas, with only very low levels measured in emus (Hackstein and Van Alen 1996). Therefore, the authors of that study classified emus as non-producers. This is in contrast to the estimation of CH_4 production of the Australian National Inventory Report (ANIR 2009), where the same daily amount of CH_4 is assumed for ostriches and emus. Recent methane measurements in adult ostriches documented a much higher CH_4 production than previously assumed in the literature, to the effect that adult ostriches produce similar amounts of CH_4 as expected for similar-sized non-ruminant mammalian herbivores (Frei et al. 2015a).

Based on these findings and the considerations outlined above, we hypothesized that rheas produce less CH_4 than expected for similar-sized ostriches and, in general, non-ruminant mammals, and that emus produce even less. For that purpose, an experiment was conducted where ratite species were compared based on the same diet and with the same respiration chamber equipment.

2. Materials and methods

2.1. Experimental design, feeding, housing and sample collection

The experiments took place after approval by the Swiss Cantonal animal care and use committee (animal experiment license no. 142/2011). Details of measured food intake, digestibility and digesta retention in ostriches, rheas and emus have been reported in Frei et al. (2015b). The experiment was performed in summer 2013 in central Switzerland, with ambient temperatures ranging between 8°C at night and 32°C during the day. Six adult rheas (body mass [BM] 23.4 ± 1.9 kg) and two adult emus (BM 33.5; 32.0 kg) were available for the present study from a private collection. All animals received a diet exclusively consisting

of pelleted lucerne (*Medicago sativa*). Additions of minerals and vitamins were made before pelleting, which was achieved under steam-heating. The nutrient composition of the pellets as analysed during this study (see Frei et al. 2015b for methods) is listed in Table 1. Pellets and water were provided *ad libitum* in any experimental phase, and no access to other food items was given. The animals were weighed once at the end of the experiment on a mobile scale.

The experiment consisted of an adaptation period of 14 days (on enclosures covered with soil and woodchips but without vegetation that the animals could consume in addition to the offered diet), 7 days of collection and 1 day of respiration measurements. For the last 3 days of the adaptation period and the 7-day collection period, the animals were kept individually in sheltered outdoor enclosures of a size of 12 m^2. Although kept individually, they had access to visual, acoustic and – through the enclosure fencing – also physical contact with conspecifics. The enclosures were protected against direct sunlight, rain and wind, and the floors were covered with fabric carpets to facilitate faecal collection. All animals were habituated to human presence.

2.2. Respiration measurements

At the end of the 7-day collection period, animals were moved individually for 23 h into respiration chambers (1.7 × 1.3 × 1.7 m). The two chambers were custom made on site out of wood, with a fabric carpet flooring. Any gaps were covered with construction tape or sealed off with silicon. Windows of a size of 17 × 42 cm, made of acrylic glass, allowed the observer to constantly monitor the animals in the chambers. Water and pelleted lucerne were provided *ad libitum* and ambient temperatures ranged from 14°C to 32°C, which corresponds to the thermoneutral zone of emus (Maloney and Dawson 1994) and is close to that of ostriches (Crawford and Schmidt-Nielsen 1967); to our kowledge, the thermoneutral zone of rheas has not been determined. Chambers were constantly and unidirectionally ventilated by a pull through system. Ambient air entered the chamber through a series of air inlets at the bottom, mixed with the air within the chamber and was then pulled out through a series of air outlets on the roof by a pump (Flowkit 100, Sable Systems, Las Vegas, USA) which generated a constant airflow of 21 to 30 L/min for rheas and 86 to 90 L/min for emus. Flow and composition of outgoing air and composition of ambient air (as baseline) were alternately measured in 90 s intervals. Gas concentrations were measured by O_2 and CO_2 analysers (Turbofox, Sable Systems) as well as by a CH_4 analyser (MA-10, Sable Systems). Data were adjusted for barometric pressure, water vapour pressure and air flow rates, which were constantly recorded during respirometry (Turbofox, Sable Systems). The gas analysers were

calibrated prior to each measurement by using pure N_2 gas and a span gas (PanGas, Dagmarsellen, Switzerland; 19.91% O_2, 0.51% CO_2 and 0.49% CH_4 dissolved in N_2). While gas recovery could not be tested due to the nature of the on-site chambers, measurements taken with this system showed a high degree of correspondence to literature data for oxygen consumption in various species (Dittmann et al. 2014; Frei et al. 2015a; Hagen et al. 2015; Vendl et al. 2015), supporting reliability of the data. In particular, a putative restriction in gas recovery would mean that O_2 consumption measurements represent over-, and CH_4 emission measurements represent underestimates, which would not change the qualitative relevance of our findings. Data obtained by the respiratory system were analysed with the software ExpeData (Sable Systems) for O_2 consumed and CO_2 as well as CH_4 emitted after correcting for gas concentrations in ambient air. To calculate the overall metabolic rate (MR) per individual, the amount of O_2 consumed (in L) was multiplied by 20.08 kJ/L (based on McNab 2008). This approach accounted for the entire time the animals spent inside the respiration chamber and therefore includes all activities by the animals inside the chamber (e.g. standing, resting, feeding). The resting metabolic rate (RMR) was estimated by selecting the 20 lowest O_2 measurement data points of each animal within the 23-h period (adapted from Derno et al. 2005). Volume measures of CH_4 were transformed into energy using the conversion factor 39.57 kJ/L (Brouwer 1965).

2.3 Comparative data sources and statistical analyses

Comparative data on the O_2 consumption of rheas and emus were taken from the literature (Crawford and Lasiewski 1968; Taylor et al. 1971; Calder and Dawson 1978; Maloney and Dawson 1993; Maloney and Dawson 1994). Ostrich data were taken from Frei et al. (2015a). For further comparisons, the regression equations for ruminant and non-ruminant mammalian herbivores described by Franz et al. (2011) were used. For the evaluation of the influence of digesta retention and relative food intake on measures of CH_4 production, measurements in the same bird individuals from Frei et al. (2015b), and data for sheep and ponies from Franz et al. (2010) were added. Simple correlations were tested by Spearman's rho (ρ). A General Linear Model was performed to analyse whether body mass, relative dry matter intake (DMI) and mean retention time (MRT) influenced the daily CH_4 output; normal distribution of residuals was ascertained to validate the approach. Analyses were performed in SPSS 21.0 (SPSS Inc., Chicago, IL). The significance level was set to $P<0.05$, with values up to 0.01 considered as trends.

3. Results

The daily pattern of O_2 consumption and CO_2 production, as displayed exemplarily for one rhea and emu each (Fig. 1A,B), indicate a high activity soon after the beginning of respiratory measurements, with a concomitant increase in CH_4 emission. The plateau indicating a night-time resting period was more distinct in the rheas than in the two emus (Fig. 1A); a change of the respiratory quotient during the night time period supports behavioural observations made during the digestion study that animals did not feed at night. Although emus had higher absolute levels of CH_4 emission, rheas had higher levels per unit food and energy intake (Table 2). The resting metabolic rates for rheas and emus were 182 ±19 and 240 ±25 kJ/kg$^{0.75}$/day, respectively (Table 3).

Compared to regression lines for ruminant and non-ruminant mammalian herbivores, all three ratites produced CH_4 of a magnitude expected for a similar-sized non-ruminant mammal (Fig. 2a). Considering CH_4 production per unit ingested dry matter or gross energy, the predicted sequence with ostriches having highest and emus lowest values was visible, but values were generally also of a magnitude observed in non-ruminant mammals (Fig. 2b,c). The CH_4 per unit digested fibre showed no evident ranking between the three ratite species (Fig. 2d).

There was no significant correlation between the MRT of particles in the gastrointestinal tract and the amount of CH_4 produced per unit body mass, neither for the ratite data alone nor in combination with the mammal data (Fig. 3A). In contrast, there was a significant correlation between the MRT and the amount of CH_4 produced per unit DMI, irrespective of whether or not mammals were included (Fig. 3B). In the dataset comprising ratites and mammals, a general linear model with absolute daily CH_4 production as the dependent variable and body mass, relative DMI and MRT of particles as covariables, the body mass effect was only apparent as a trend ($F = 4.150$, $P = 0.060$), whereas the effects of both relative DMI ($F = 11.197$, $P = 0.004$) and MRT ($F = 42.003$, $P < 0.001$) were significant.

4. Discussion

The results of the present study demonstrate substantial CH_4 production in rheas and emus. In comparison with the findings in ostriches, these results suggest that CH_4 production depends on both, food intake and digesta MRT in the gastrointestinal tract - within ratites, and possibly also across other herbivorous vertebrates. The interplay between intake, retention time and CH_4 production might suggest that the absolute daily CH_4 production may be similar for animals that have a high food intake but a short retention time vs. animals that have a

lower food intake but a longer retention time. This means that, in order to classify animals in terms of CH_4 production, the focus should not only be on CH_4 'yield' (per unit food intake or energy intake, or per unit faecal material) but also on the overall daily CH_4 emission.

The metabolic rate measurements in the emus corresponded to resting metabolism as measured by chamber respirometry in another study, but were below levels determined for standing metabolism by mask respirometry, and higher than the basal metabolic rate measures determined in respiration chambers (Table 3). For rhea, only mask respirometry measurements are available in the literature; in one study, the presumed resting metabolism was higher than the standing metabolism of another study (Table 3). The resting metabolism measured in the rheas of the present study was lower than that measured in the emus, and was within the range reported as basal metabolic rate for emus (Table 3).

Although the diet fed to the animals in this study was artificial insofar as it consisted of dried, ground and pelleted plant material, it can be considered representative for the natural diet of the species - in the case of the emu, at least for the natural diet of certain parts of the year (reviewed in Frei et al. 2015b). Evident differences to the natural diet in term of moisture content were could be compensated for the animals by using the offered drinking water, and animals were regularly observed doing this. Compared to herbivorous mammals, herbivorous birds achieve a similar degree of ingesta particle size reduction with their gizzard (Fritz et al. 2011). In cattle, pelleting lucerne hay led to a slight reduction of CH_4 production as compared to feeding the same material in a chopped chopped state (Hironaka et al. 1996). Therefore, an undue overestimation of the CH_4 production in ratites because of the diet used in the present study appears unlikely.

In addition to the finding that *in vivo* CH_4 measurements in ostrich contradicted several *in vitro* assessments that suggested an absence of CH_4 emission in this species (Frei et al. 2015a), the *in vivo* findings in the two emus of the present study in addition contradict the classification of emus as non-producers (Hackstein and Van Alen 1996). One reason for this contrasting finding could be that Hackstein and Van Alen (1996) reported the low CH_4 production of the emus in relation to units of faecal mass. Given the findings of the present study, with low measured CH_4 emissions per unit of DMI in this species, the low CH_4 production from faeces appears plausible; however, due to the particularly high food intake (and high faecal output) of emus, high overall CH_4 emissions per day occurred nevertheless. Thus, the findings on ratites from Frei et al. (2015a) and the present study emphasize the importance of not only relying on *in vitro* measurements of proxies of CH_4 production, but of corroborating such findings by *in vivo* measurements.

Within domestic sheep (Hammond et al. 2013), goats (Aguilera and Prieto 1991) and cattle (Yan et al. 2010), negative relationship exist between CH_4 expressed as proportion of DMI or gross energy intake (CH_4 'yield') on one hand and the food intake level on the other hand. Presumably, the reason for this is the negative effect of intake on digesta retention time (Clauss et al. 2007; Munn et al. 2008). Correspondingly, Pinares-Patiño et al. (2003) found that CH_4 yield increased across treatments with digesta retention in the rumen. In the same line of evidence, Goopy et al. (2014), Hammond et al. (2014) and Barnett et al. (2015) found that CH_4 per unit of DMI increased in sheep with increasing rumen MRT. The general similarity of these findings with the results of the present study might suggest that the mechanism by which retention time influences CH_4 per unit of DMI is quite similar both within and across species. Thus, ruminants may generally produce higher levels of CH_4 than many other mammals due to the fact that their digestive strategy allows a comparatively high food intake at comparatively long retention times (Clauss et al. 2010), which is associated with an increased fibre digestibility due to their enhanced digesta particle size reduction (Clauss et al. 2015).

These results represent an expansion of the mechanistic understanding of CH_4 production, which was so far based on studies within ruminant species, by our study of ratite species: it seems that even at extremely short retention times, such as those observed in emus (Fig. 3AB), a certain amount of CH_4 production takes place that appears to be linked to the digestion of fibre (Fig. 2D). This appears counter-intuitive, because CH_4 production is mainly associated with long time periods (Prins and Kreulen 1991; Van Soest 1994). However, generation times of as low as 1 h have been reported for certain methanogens (Thauer et al. 2008). The findings of the present study might point towards an interpretation that there are no specific evolutionary adaptations in herbivores that allow or prevent the presence and action of methanogens (sensu Hackstein and Van Alen 1996), but that these are subject to effects of other components of digestive strategy such as food intake level and digesta retention. An interesting question is whether principally the same type of Archaeal community is involved in any CH_4 production in herbivores regardless of the time available, whether their output rate is then simply limited by the amount of hydrogen ions supplied by fibre-digesting microbes, whether their metabolic state is different under such conditions limiting CH_4 production (Shi et al. 2014), or whether different Archaeal communities are involved in CH_4 production at different levels of digesta retention, such as 2 h vs. 40 h. Molecular analyses of gut contents of ostriches vs. emus could help answer this question.

Acknowledgements

We are grateful to Max Kleinpeter for access to rheas and emus, Carmen Kunz, Muna Merghani, Elisabeth Wenk and Heidrun Barleben for sample analysis. This study was part of project 310030_135252/1 funded by the Swiss National Science Foundation and by the Basle Foundation for Biological Research. We thank David Wilkinson and an anonymous reviewer for comments on the manuscript.

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363
364

365 **Table 1** Nutrient composition of the lucerne
366 pellets[a] used in the present study

Nutrient	Unit	
Organic matter	[g/kg DM]	883
Crude protein		177
Ether extract		21
Neutral detergent fibre		418
Acid detergent fibre		330
Acid detergent lignin		77
Gross energy	[MJ/kg DM]	18.0

367 [a]Product no. 2805, Provimi Kliba SA,
368 Kaiseraugst, Switzerland
369 *DM* dry matter
370

371 **Table 2** Body mass, food intake, apparent digestibilities and methane production
372 in rheas (*Rhea americana*) and emus (*Dromaius novaehollandiae*) fed lucerne
373 pellets

Measure	**Unit**	**Rhea (n=6)**	**Emu (n=2)**
Body mass (BM)	[kg]	23.4 ±1.9	33.5; 32.0
Dry matter intake (DMI)	[g/kg$^{0.75}$/day]	59 ±26	263; 195
Apparent digestibility of			
Dry matter		55 ±7	42; 47
Neutral detergent fibre	[%]	43 ±10	22; 28
Gross energy		54 ±7	45; 49
Methane	[L/day]	2.87 ±0.82	7.39; 6.25
	[L/kg BM/day]	0.13 ±0.04	0.22; 0.20
	[L/kg DMI]	5.2 ±2.4	2.1; 2.4
	[% GEI]	1.2 ±0.6	0.5; 0.6
	[% DEI]	2.2 ±0.9	1.1; 1.1
	[L/kg dNDFi]	31.1 ±11.8	23.9; 21.5

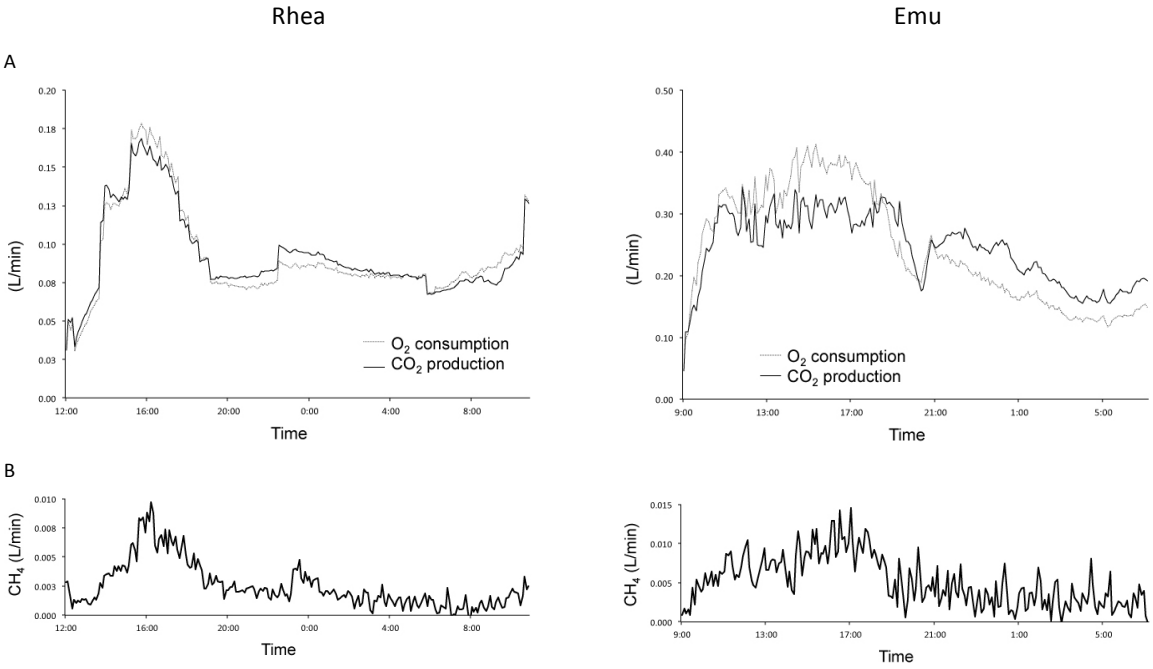
374 *GEI* gross energy intake, *DEI* digestible energy intake, *dNDFi* intake of digestible
375 neutral detergent fibre

376 **Table 3** Metabolic rate measurements based on oxygen consumption (mean ±SD) in rheas (*Rhea americana*) and emus (*Dromaius novaehollandiae*)

Species	**n** (male/female)	**Diet**	**BM** [kg]	**RQ**	**MR**	**SMR**	**RMR**	**BMR**	**Method of respirometry**	**Reference**
					---------- [kJ/kg$^{0.75}$/day] ----------					
Emu	2	L	33 ±1	1.04 ±0.05	455 ±56		240 ±25		Chamber	Present study
	4	N / T	39 ±4				242 ±23		Chamber	(Calder and Dawson 1978)
	5/5 (winter)	Mix	37/40					180/224	Chamber	(Maloney and Dawson 1993; Maloney and Dawson 1994)
	5/5 (summer)		41/45					177/221		
	2	Mix	38			282			Mask	(Crawford and Lasiewski 1968)
Rhea	6	L	23 ±2	0.95 ±0.06	231 ±23		182 ±19		Chamber	Present study
	3	Mix	22			329			Mask	(Crawford and Lasiewski 1968)
	2	-	22				397		Mask	(Taylor et al. 1971)

377 *BM* body mass, *L* Lucerne, *N* natural foraging, *T* commercial turkey diet, *Mix* mixed diet of pellets, fruits, lettuce and bread, *RQ* respiratory quotient
378 calculated as CO_2/O_2, *MR* metabolic rate, *SMR* standing metabolic rate, *RMR* resting metabolic rate, *BMR* basal metabolic rate

379



380 **Figure 1** Examples of the results of the respiration measurements for A) oxygen and carbon
381 dioxide and B) methane in one rhea (*Rhea americana*) and one emu (*Dromaius*
382 *novaehollandiae*) individual

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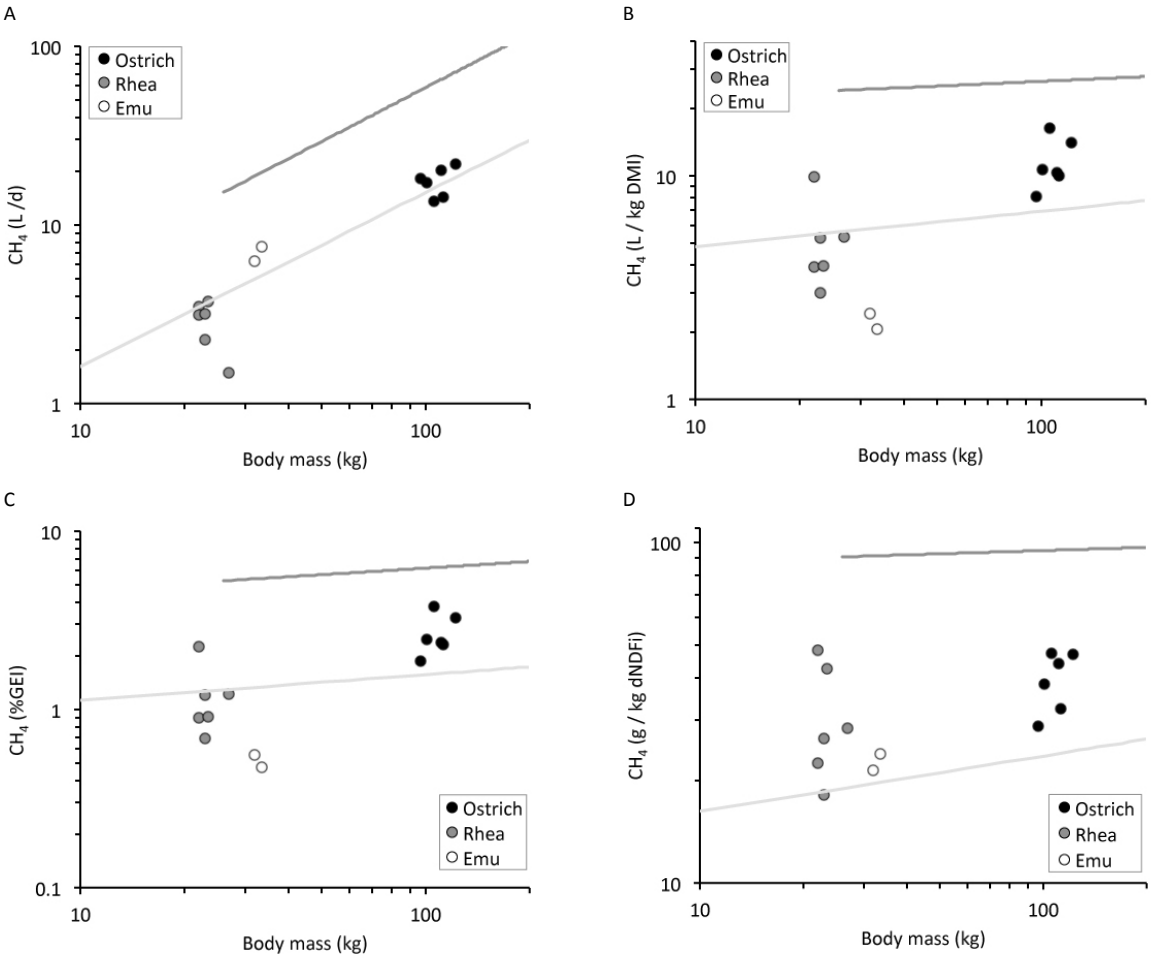

385 **Figure 2** Comparison of the methane emission by adult ostriches (*Struthio camelus*; data
386 taken from Frei et al. (2015a)), rheas (*Rhea americana*) and emus (*Dromaius*
387 *novaehollandiae*; both from the present study) with data from ruminants (dark grey line) and
388 non-ruminant mammals (light grey line) (Franz et al. 2011). Methane presented as A)
389 absolute daily amounts, B) per unit of daily dry matter intake (DMI), C) percentage of gross
390 energy intake (GEI) and D) per unit of digestible neutral detergent fibre intake (dNDFi)

391

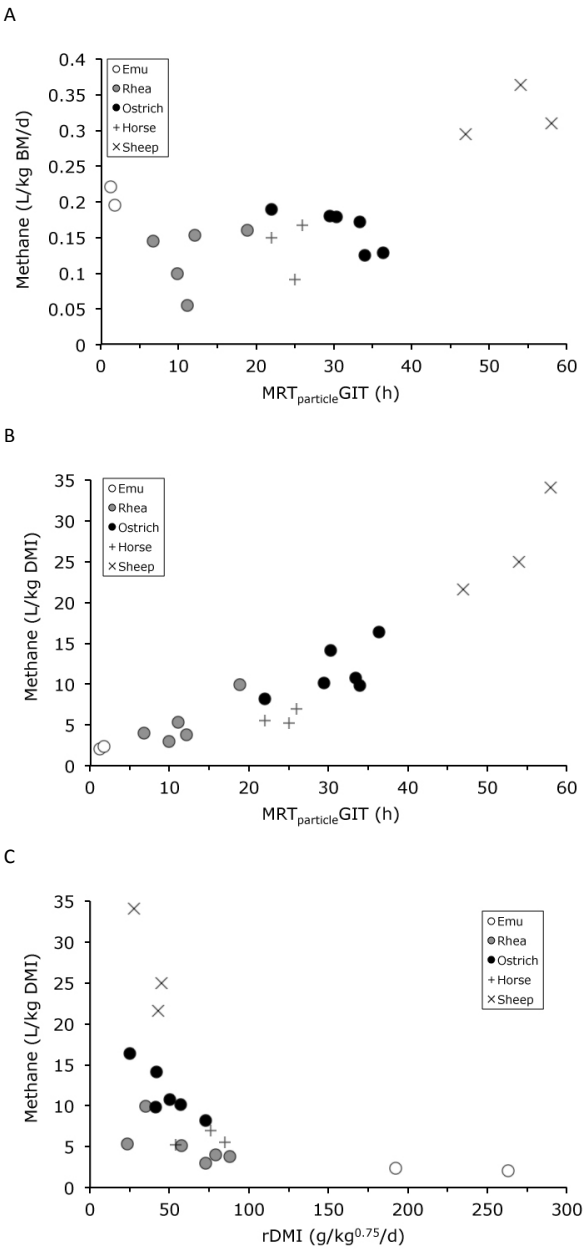

Figure 3 Combined presentation of data on CH_4 production and dry matter intake (DMI) intake in adult ostriches (*Struthio camelus*; taken from Frei et al. (2015a)), rheas (*Rhea americana*) and emus (*Dromaius novaehollandiae*; both from the present study) with data on mean retention time (MRT) of particles in the gastrointestinal tract (GIT) obtained in the same individuals (data taken from Frei et al. (2015b)) and data on sheep and ponies (taken from Franz et al. (2010)). Results of correlation analysis: A) between MRT and CH_4 produced per unit of body mass: ratites: ρ = -0.236, P = 0.437; ratites and mammals: ρ = 0.315, P = 0.189; B) between MRT and CH_4 produced per unit of daily DMI: ratites: ρ = 0.901, P < 0.001; ratites and mammals: ρ = 0.928, P < 0.001; C) between the relative DMI and CH_4 produced per unit of DMI: ratites: ρ = -0.789, P = 0.001; ratites and mammals: ρ = -0.734, P < 0.001